THE EFFECT OF PHAGOCYTOSIS ON THE GROWTH AND SURVIVAL OF AVIAN TUBERCLE BACILLI IN EMBRYONIC CHICKEN TISSUE CULTIVATED *IN VITRO*

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(With Plates 1–3)

INTRODUCTION

One method by which the host:parasite relationship between animal cells and tubercle bacilli may be investigated is by inoculating cultures of tissues from resistant and susceptible hosts with bacterial strains of different degrees of virulence. Experiments of this type have been described by Smyth (1916), Smith, Willis & Lewis (1922), Maximow (1924, 1925*a*, *b*, 1926, 1928), Lang (1925) and Timofejewsky & Benewolenskaja (1925, 1926, 1927, 1928), Haagen (1927).

These investigations suggested that tissue grown *in vitro* reacts similarly to tissue *in vivo*, and that the type of reaction depends upon species pathogenicity and virulence. When tissue cultures were infected with a non-virulent strain, e.g. adult rabbit tissue inoculated with the BCG strain (Maximow, 1928) or with non-virulent human-type bacilli (Maximow, 1924, 1925*a*, *b*, 1926; Timofejewsky & Benewolenskaja, 1925), a kind of symbiosis was usually established between the animal cells and the bacilli; under such conditions many organisms were ingested by the wandering cells and a few by the fibroblasts, while others grew profusely over the tissue which appeared unharmed by the association. The macrophages reacted to the infection by forming clumps resembling early tubercles, by fusion into multinucleate giant cells of the Langhans type and by certain characteristic cytological changes; there was comparatively little necrosis in the tissue which was eventually obliterated by the mere overgrowth of the organisms. On the other hand, when cultures were infected with virulent bacilli of a type to which the tissue was susceptible, e.g. human embryonic tissue infected with a virulent human strain (Timofejewsky & Benewolenskaja, 1928) or adult rabbit tissue inoculated with a bovine strain (Maximow, 1928), degeneration and necrosis were seen.

Phagocytosis was shown to be the main factor in the control of infection. There is, however, no conclusive evidence in the literature about the fate of the ingested tubercle bacilli and their survival in tissue cells under pathogenic and non-pathogenic conditions. In Maximow's experiments with cultures of adult rabbit tissue infected with a virulent bovine strain, the macrophages showed no sign of digesting the phagocyted bacilli, but were themselves killed by the organisms which continued to multiply after the death of the cell. When, however, tissue cultures were infected with either non-virulent bacilli or bacilli of a type to which the tissue was partially resistant, the results varied. Smyth (1916), describing the reaction *in vitro* of embryonic chicken spleen to human-type bacilli, states that cells which have phagocyted the organisms 'may digest them or may be unable to do so, in which case the bacilli develop within the cells'. Similarly, Maximow, after inoculating cultures of adult rabbit tissue with an actively growing human strain of low virulence, found that in some cells the ingested bacilli showed 'unmistakable signs of intracellular digestion', while 'in other cells of the same culture the engulfed bacilli proliferate in the body of the phagocyte' (Maximow, 1924). In other rabbit cultures inoculated with a more slowly growing but (to guinea-pigs) virulent strain of human bacilli 'the cells of the rabbit tissues overpower and destroy them rapidly' (Maximow, 1924). Similar results were obtained by Timofejewsky & Benewolenskaja (1925). In rabbit tissue cultures lightly infected with BCG, the cells engulfed and apparently digested the bacilli which eventually completely disappeared from the tissue, but the wandering cells were not sufficiently numerous to master a heavy infection which finally overgrew the entire culture (Maximow, 1928). Smith *et al.* (1922) described beautiful warm-stage observations on embryonic avian tissue cultures in which the ingested bacilli gradually disintegrated into small granules; these authors used avian tubercle bacilli of low virulence and a tissue culture medium which did not permit active bacillary growth.

All the foregoing accounts of the intracellular destruction of bacilli in tissue cultures were based

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on morphological evidence only, and no attempt was made to find whether the phagocyted organisms were capable of growth if the host cell were killed and the bacilli provided with a suitable nutrient medium.

The present investigation was planned with two main objects: first, to study some of the factors on which the control of infection in tissue cultures depends, and secondly, to obtain more precise information about the viability of ingested bacilli of virulent and non-virulent strains. The avian type of infection was chosen for the following reasons: (1) the avian bacillus is a natural parasite of the fowl, (2) avian embryonic tissue grows very rapidly and vigorously *in vitro* and can be readily cultivated in homologous medium in which the avian bacillus also grows profusely. The experiments have been confined to the one type of infection, so that the behaviour of several strains of the same type but of varying pathogenicity could be compared. The capacity for growth of bacilli ingested by individual cells has been investigated by a bacteriological viability test specially devised for the purpose.

MATERIAL AND METHODS

Bacterial strains. Five avian strains were used:

(1) 'Bone' (B strain) recovered from the bone marrow of a chicken which had died from tuberculosis and which was kindly sent to us from the Weybridge Veterinary Laboratory. The primary culture on slants of Löwenstein's medium grew slowly but subcultures grew well, forming irregular granular colonies. Tissue explants were inoculated with suspensions from both the primary cultures and the subcultures. The strain was found to be very pathogenic, killing rabbits 6 weeks after peritoneal injection (Yersin type of infection).

(2) 'Weybridge' (W strain) received from Weybridge Research Institute; it had been isolated from a tuberculous bird in October 1944, inoculated on Dorset agar and subcultured on glycerin potato agar. Its pathogenicity for rabbits was moderate; intraperitoneal injection produced caseous foci and intravenous injection killed adult rabbits.

(3) 'Original' (O strain). Its history has been described in a previous publication (Brieger & Fell, 1945). This strain, which has been subcultivated on Löwenstein's medium for 4 years, has now lost much of its pathogenicity, and of the four rabbits infected with it only one (an albino) died after intravenous injection.

(4) 'Lister' (L strain), received from the National Collection of Type Cultures on glycerin agar in 1943, and described by Brieger & Fell (1945). Its pathogenicity, always slight, has now entirely gone; rabbits survived indefinitely after intravenous injection and when killed showed no lesions.

(5) 'Amnion Fluid' (AF III strain). A derivative of the 'Original' strain which was recovered from the amnion fluid of a foetal guinea-pig *in utero*, 3 days after intra-amniotic infection. At a time when the mother strain was still highly pathogenic for fowls, the AF III strain, as a result of its intra-amniotic passage, had completely lost its pathogenicity and when injected into fowls produced no foci. It grows very profusely and rapidly on Löwenstein's medium, is very polymorphic and forms a proportion of branching forms.

Tissue culture. Most of the experiments were made on lung tissue from 11–12-day chick embryos. Usually about eighteen explants of suitable size were provided by each pair of lungs. Cultures of 11–12-day embryonic frontal bone were also used for special purposes.

All cultures were made by the hanging-drop method on $1\frac{1}{4}$ in. square cover-slips sealed with paraffin wax over $3 \times 1\frac{1}{2}$ in. hollow-ground slides. The medium consisted of equal parts of fowl plasma and 11–12-day chick embryo extract prepared by mixing 1 part of ground embryo mince with 2 parts of Tyrode's solution containing 0·1 % glucose. To infect the cultures, the medium was allowed to clot and a small droplet of the bacterial suspension was carefully deposited on the explant. After being sealed, the cultures were incubated upside down overnight to allow the bacilli to deposit on the tissue and surrounding medium. The incubator was kept at 37·5–38° C.

At 3-day intervals the explants were subcultivated by excision from the clot, washing in saline and transference to fresh plasma and embryo extract. In many experiments we wished to preserve intact the areas of heaviest infection. To do this we examined the cultures under a low-power objective and made an Indian ink mark on the glass over the most densely infected region in each culture; this region could then be identified while the explant was being cut from the clot and subdivided, and if necessary could be kept untouched for several passages. In other experiments the tissue was subdivided at random in the usual way.

Bacterial viability tests. A method was devised for studying the viability of phagocyted bacilli in infected tissue cultures. To sterilize the outer surface of the cover-slip, the complete hanging-drop preparation was first immersed in 5 % oxalic acid and incubated at 37·5° C. for about half an hour. The cover-slip was then removed aseptically from the hollow-ground slide and laid face upwards in a sterile Petri dish which was placed in the incubator; when the culture was dry, the cover-slip was inverted on to a Löwenstein plate which had been liberally flooded with sterile distilled water. The Petri dish was sealed with plasticine and incubated.

This method killed but did not disarrange the

cells of the tissue culture, while viable bacilli were unaffected and grew very rapidly in contact with the Löwenstein's medium.

Fixation and staining. The cultures were fixed for 2–3 min. in Zenker's fluid without acetic acid from which they were transferred direct to 80% alcohol where they remained overnight to ensure complete sterilization of the entire cover-slip. They were then placed in alcoholic iodine solution for about 1 hr., returned to clean 80% alcohol for 1–2 hr. and finally washed in distilled water for 1–2 hr. After the paraffin wax had been removed with a safety-razor blade, the cultures were stained with carbol-fuchsin for 2–5 min. on a hot plate, decolorized in the usual way and placed for 20 min. in Ehrlich's haematoxylin. After being 'blued' in two changes of tap water for about an hour, the preparations were dehydrated, cleared in xylol and mounted whole in Canada balsam. This technique gave good fixation and a very clear picture of both cells and bacilli. The shortness of the fixation period prevented the plasma clot from staining too densely and obscuring the growth.

Since explants of embryonic chick tissue spread into relatively thin sheets and liquefy the clot above them, sufficient detail could be seen in the interior of the tissue in the whole mounts. A few cultures, however, were serially sectioned for special purposes.

RESULTS

I. *The control of avian tubercle infection in embryonic lung cultures*

These observations were made on several hundred lung cultures prepared in the course of the research. Explants were infected with each of the five bacterial strains, and the reaction of the tissue to all five strains was essentially the same.

Immediately after explantation the infected lung fragment appeared as a compact lump bulging from the clot in which it was embedded and surrounded by a pool of bacterial suspension resting on the surface of the medium. After the culture had been incubated upside down for 16–18 hr., most of the bacilli in the droplet had settled on the clot. At the same time the explant, which had begun to flatten and spread, had formed a narrow marginal fringe of amoeboid fibroblasts, among and beyond which wandered many macrophages and monocytes busily ingesting the bacilli (Pl. 1, fig. 1). Some of the macrophages were already so stuffed with bacilli as to have a golden brown colour when seen by transmitted light.

During the next 24 hr. the explant rapidly expanded into a thin sheet in which ran the branching epithelial tubules. The engorged macrophages wandered over the lower surface of the zone of outgrowth, often forming long, crescentic tracts of brownish cells frequently aggregated in small nests and clusters and staining brilliantly with carbol-fuchsin (Pl. 1, fig. 2). Other infected wandering cells migrated into the interior of the explant, into the somewhat dilated vascular spaces, into the epithelial tubules, or far into the plasma clot beyond the fibroblastic outgrowth. Some fell into the droplet of serous fluid which always forms beneath the growing tissue, and there aggregated into large, freely drifting masses. Large multinucleate giant cells like those described by previous workers (Smyth, 1916; Maximow, 1924; Timofejewsky & Benewolenskaja, 1925) developed in these aggregates by fusion of uninuclear elements. Such giant cells were less common elsewhere in the cultures. If transferred with a pipette to fresh medium the loosely drifting wandering cells became amoeboid and proliferated actively.

By the end of the second or third day the tissue had expanded over the whole infected area of clot, unless the droplet of suspension was unusually widespread, and apart from a few organisms scattered in the fluid exudate beneath the tissue, all the free bacilli had been phagocyted by the wandering cells which now swarmed all over the culture and surrounding medium in vast numbers. Some fibroblasts and, where a tubule had spread into the zone of outgrowth, epithelial cells also contained bacilli but in relatively small numbers, and the wandering cells were mainly responsible for mastering the infection. Cell division was active, and even cells containing many bacilli of a virulent strain might undergo mitoses.

During subculture some of the infected macrophages were lost either because they fell into the fluid beneath the explant or because they crawled far into the clot, so that the number of infected cells declined somewhat after the first 3 days' growth. The main tracts of heavily engorged cells embedded in the tissue, however, were often preserved throughout the culture period (see section on technique). They could be studied during several passages without any extensive cell degeneration being observed, and in stained preparations the nuclei of most of the engorged cells appeared normal even when the bacilli were from a virulent strain. Provided the culture was healthy and growing well, free bacilli were rare, and the few encountered had been liberated by the degeneration of an isolated cell. Such liberated bacteria seemed to be rapidly engulfed by neighbouring wandering cells and stages in the phagocytosis of infected cell debris were seen in the stained preparations. In actively growing cultures there was no gross increase in the amount of infection for 2 and sometimes 3 weeks, but it is uncertain whether a slight increase took place, as this would have been undetectable.

After the first few days of cultivation the ingested organisms became less refractile. The golden brown colour which characterized the densely infected macrophages in the young cultures viewed by transmitted light gradually faded to a silvery hue and the cells were much less conspicuous than before. In stained preparations acid fastness diminished conspicuously in ingested bacilli of the AF III strain, slightly in those of the L and B strains, but remained intense in those of the O and W strains, even in cultures maintained for 2–3 weeks. As stated above, the AF III organisms are not very strongly acid-fast under normal conditions of growth, so that loss of acid-fast material in this strain might be more readily shown by Ziehl-Neelsen's method than in bacilli which are more richly endowed with the acid-fast substance.

Conclusions (§ I)

(1) During the first two weeks of growth cultures of embryonic avian lung appear unharmed by infection with avian tubercle bacilli of either virulent or avirulent strains.

(2) Such cultures can control infection, both virulent and avirulent, for 2–3 weeks by the phagocytic activity of their many wandering cells.

II. *The limiting factors in the control of infection by tissue cultures*

(1) *The effect on control of reducing the volume of lung tissue relative to the amount of infection*

In one of the many experiments made on infected lung explants, the infection was not controlled in all cultures in the manner described in § I. This experiment was done in winter, and the only embryo available was very small for its age (a common condition in winter eggs). To provide enough cultures much smaller explants than usual were made. Half were infected with the original culture of the virulent B strain which grew slowly, having been recently isolated from the fowl, and the rest with the profusely growing but avirulent L strain.

During the first 36 hr. the outgrowth from all the explants was almost entirely fibroblastic so that the bacilli were able to multiply over and around the tissue with little interference from wandering cells. In the series infected with the L strain, the fibroblasts pushing through the proliferating masses of free bacilli ingested far more bacteria than usual and many became heavily infected. Between the second and third day the wandering cells began to pour out of the explants, multiplied rapidly and voraciously devoured the bacilli, so that by the end of the third day in most of the cultures nearly all the free organisms had been phagocyted. The engorged macrophages were more distended than in experiments where the proportion of lung tissue to bacilli was greater. During subsequent cultivation these swollen wandering cells remained dilated, retained their yellowish colour when viewed by transmitted light and formed large clumps and tracts among heavily infected fibroblasts. As the culture period advanced, some of these grossly infected areas enlarged while the density of the bacilli noticeably increased, showing that such a large bacillary content was beyond the complete control of the tissue.

The series of lung cultures infected with the more slowly growing, though far more virulent B strain behaved differently. The number of bacilli incorporated in the tissue was much less and the wandering cells were not greatly swollen. During subcultivation the ingested organisms lost their yellowish colour and no spread of the infection could be detected.

This accidental observation was investigated further. Two series of lung cultures were made, in one of which the explants were of the usual size, i.e. about 2 mm. square, and in the other very much smaller; both were infected with a subculture of the B strain, now more quickly growing though still highly virulent *in vivo*. The droplets of suspension delivered with the same platinum loop were approximately the same size in both series, so that the small explants received a relatively much heavier infection than the large.

After 24 hr. incubation, many wandering cells had emerged from the larger explants and had engulfed nearly all the free bacilli, but few had migrated from the small explants which, however, appeared very healthy and showed active fibroblastic outgrowth among the proliferating masses of free bacilli. By the end of the third day the large explants had completely phagocyted the bacteria; phagocytosis having been rapid, the organisms had not had time to multiply much so that the groups of infected macrophages in the tissue were not very large and infection of the fibroblasts was relatively slight (Pl. 1, fig. 2).

At this stage of growth the small explants presented a very different picture (cf. Pl. 1, figs. 2, 3). Macrophages were now fairly abundant in the zone of outgrowth, but owing to the inadequate phagocytosis of the first 2 days the free bacilli had been able to multiply extensively before the army of wandering cells arrived. As a result, the fibroblasts were far more heavily infected than in the larger explants, and the number of engorged macrophages was much greater. During subsequent cultivation the amount of infection seemed to increase in some of the small cultures, but to a less extent than in some of those infected with the rapidly growing L strain in the previous experiment.

The results of these experiments on small lung explants showed that the control of infection

depends partly on the relation between the size of the explant and the amount of infection, and partly on the growth-rate of the bacilli. Thus the small explants in the first experiment were able to control a heavy infection of a slowly growing strain but could not always suppress a similar infection of a rapidly growing strain.

It was also interesting to see how the protective action of the wandering cells towards the fibroblasts declined when the proportion of tissue to bacilli was reduced beyond a certain level; thus the number of heavily infected fibroblasts was far greater in the small explants because wandering cells were not numerous enough to ingest quickly the free bacilli before they had time to multiply extensively over and round the emigrating fibroblasts.

(2) *The effect on the control of bacillary growth of prolonged cultivation of infected lung tissue*

Experiments were made to find whether bacillary growth could be controlled indefinitely by embryonic lung tissue *in vitro*. In the first experiment two series of lung cultures were prepared and infected in the usual way with the virulent W and the avirulent AF III strains respectively. The cultures were subdivided at intervals and maintained for 30–42 days, when they were fixed and stained.

As in earlier experiments the free bacilli in the droplet of suspension were rapidly phagocyted by swarms of wandering cells during the first 3 days' cultivation, and for several passages the infection was confined mainly to these cells though some of the fibroblasts also contained a few bacilli. In living cultures viewed by transmitted light the phagocyted organisms of the AF III strain were more conspicuous than those of the W strain and preserved their brownish colour longer.

The cultures grew very actively for the first 9 days, but after this stage the epithelium began to spread out on the cover-glass in large sheets which liquefied the medium in places and caused the fibroblastic outgrowth in the underlying clot to retract, sometimes severely, so that certain cultures had to be discarded. After 21 days (six passages) this tendency to liquefy the clot and retract began to decline, and broad zones of outgrowth, composed of a mixture of fibroblasts and dedifferentiated epithelium, were again formed and the cultures could be subdivided every few days.

In some cultures the rupture of tubules and cysts, due to dedifferentiation and migration of the epithelium, liberated into the medium cell debris and free bacilli hitherto imprisoned in the cavities of these structures. The wandering cells, which were still abundant by the sixth passage, busily ingested these free organisms. Sometimes small colonies of bacteria were also seen in the interior of the tissue where they had probably been set free by the disintegration of infected cells and had been protected from reingestion by the surrounding fibrous tissue which became quite dense after several weeks' cultivation.

By the eighth passage (30th day) the number of wandering cells had greatly diminished, a usual event in tissue after repeated subculture, and free infection both in the medium and inside the tissue began to increase rapidly and appeared in an ever larger number of cultures. The depleted force of wandering cells could no longer control the situation, although the surviving macrophages ingested bacilli until their cytoplasm was distended almost to bursting point.

At about this stage an interesting difference between the two series of cultures was noted. In the AF III set the fibroblasts in the zone of outgrowth contained few bacilli, and the vigorous bacterial growth in the culture was mainly, if not wholly, extracellular; many cultures contained compact, rounded colonies in the centre of the tissue. The infection in the W series, on the other hand, was diffuse and largely intracellular, and in a heavily infected culture the emigrating cells of the outgrowth were laden with bacilli; well-defined colonies like those of the AF III strain were not formed in the cultures, though there were many free bacilli in both the tissue and the surrounding medium.

This uncontrolled infection soon began to destroy the tissue cultures. In the AF III series the tissue was suddenly disrupted by the rapid expansion of the extracellular colonies embedded in the tissue. In the cultures infected with the W strain, though bacillary growth sometimes disrupted the tissue in a similar way, usually the process of dissolution was less abrupt. Thus, when a culture with a fairly large but very heavily infected zone of outgrowth was transferred to fresh medium, only a few cells swollen with bacilli emerged during the next passage and these often disintegrated, apparently owing to the internal pressure of the proliferating organisms in the cytoplasm; after a further passage there was no outgrowth and the cells were obliterated in a dense mass of bacilli.

Some lung cultures of both series were still growing actively 6 weeks after explantation (Pl. 2, fig. 5). By then the intracellular infection in the W series was extremely heavy (Pl. 2, fig. 6), though surprisingly many quite heavily contaminated fibroblasts underwent apparently normal mitotic division in spite of the virulence of the strain (Pl. 2, fig. 7). In both series wandering cells had nearly vanished and proliferating bacilli were scattered all over the medium and tissue. The AF III cultures usually had large internal colonies, but, as before, showed comparatively little intracellular infection.

An experiment was made to test the relative virulence of the two strains after their 6 weeks' sojourn in tissue cultures. Four of the W and two of the AF III cultures were excised and smeared on Löwenstein slopes. The AF III strain grew much more luxuriantly than the W, and by the end of the second week had formed a thick film on the medium while the W strain had produced only small scattered colonies. After 5 weeks' incubation, cloudy suspensions in distilled water were made from one W and one AF III tube. One c.c. of suspension was injected intravenously into each of four young rabbits of the same litter and of an average weight of 2000 g.; two animals were inoculated with each strain. Those infected with the W strain died after 3 and 4 weeks respectively; no macroscopic lesions were present, but very many bacilli were found in the lungs and spleen, and two cultures made from the spleen on Löwenstein's medium grew well. The two rabbits infected with the AF III strain survived in apparent health and were killed after 4 months; no macroscopic tuberculous lesions were seen, but in one animal there was a slightly cloudy peritoneal exudate which, however, proved to be sterile on culture.

An experiment was made to find whether a less massive infection could be controlled indefinitely by lung cultures where a heavy infection could not. A bacillary suspension of the W strain was prepared in the usual way, and part was then diluted with saline respectively to 10 and 1 % of its original concentration. One series of explants was infected with the original undiluted suspension, one with the 10 % and a third with the 1 % dilution. The maximum culture period was 29 days (eight passages) for series I and 35 days (eleven passages) for series II and III. At first the heavily infected series grew rather less well than the other two, but after repeated subculture and subdivision these differences disappeared. The macrophages diminished in number rather more slowly than in the previous experiment, but after 4–5 weeks' growth there were far fewer than during the early stages of cultivation. Even in the two series treated with the diluted suspensions, the intracellular infection increased greatly towards the end of the culture period; the macrophages were bloated with bacilli and many fibroblasts and epithelial cells also contained masses of organisms. In all three series the fibroblastic infection though pronounced was less dramatic than in the earlier experiment, probably owing to the presence of a larger number of macrophages which, as described above, tend to protect the tissue cells from contamination. The results showed clearly, however, that even a moderate infection may not be controlled indefinitely by embryonic lung cultures.

(3) *The effect on bacillary growth on tissue containing few macrophages*

Five experiments were made to find how bacilli would behave in cultures of a tissue which normally contains few wandering cells in proportion to other cell types. The tissue selected was the frontal bone of 11–13-day chick embryos. When fragments of these bones are explanted *in vitro*, the outgrowth consists mainly of osteoblasts, and although a variable number of wandering cells also emerge, in our experience they are always much fewer than in lung cultures derived from the same embryo. The number of lymphoid cells in the outgrowth may be reduced still further by repeated subculture, though a few persist in the interior of the culture even after four or five passages.

Both original explants taken direct from the chick and cultures which had passed through several passages *in vitro* were used for these observations. Essentially the same results were obtained whether the bone were infected immediately after explantation or after repeated subculture.

The amount of phagocytosis which occurred in a given set of osteoblast cultures depended on the number of wandering cells present and also, as in the small lung cultures (see p. 362), on the growth-rate of the bacilli. Thus in Exp. 1 the bone fragment produced an unusually large number of wandering cells, the B strain with which the tissue was infected grew at only moderate speed and after 3 days' incubation six of the twelve cultures had phagocyted nearly all the free bacilli. None of the bone explants, however, produced nearly so many wandering cells as did the control lung cultures, all of which had phagocyted the free bacilli by the end of the 2nd day. On the other hand, in Exp. 4 very few wandering cells emerged from the bone, the O strain with which the cultures were infected grew profusely, and after 3 days in most cultures there were masses of free bacilli proliferating over the osteoblastic outgrowth.

Where free bacilli rested on the outgrowth, many of those in immediate contact with the cells were ingested, so that broad tracts of tissue were seen in which the cytoplasm of the osteoblasts was crowded with bacilli like that of the fibroblasts in the old lung cultures (see p. 363). This occurred in all the five experiments but was particularly striking in many bone cultures of Exp. 4. The fibroblasts of the control lung cultures, made from the same embryo as the bone explants, incorporated far fewer bacilli than did the osteoblasts owing to the protective action of the swarms of wandering cells which the lung contained at this stage of cultivation.

After several subcultures the differences between the infected bone and lung explants became even more conspicuous (cf. Pl. 2, figs. 8, 9). Most of the free bacilli in the bone cultures were washed away

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at the first transplantation, but those in the infected tracts of the outgrowth continued to multiply so that the tissue became brownish and rather opaque when viewed by transmitted light. For a time the osteoblasts seemed hardly affected by their heavy bacterial content even when the organisms were from a virulent strain; osteogenic fibres were formed and the osteoblastic nuclei appeared normal. A few infected macrophages, enormously engorged with bacteria, were usually present in the brownish areas, and in preparations stained by Ziehl-Neelsen's method appeared as irregular, deep crimson blotches scattered about the infected region. In the control lung cultures the bacilli were still mainly concentrated in the wandering cells, and there was no visible increase in the infection.

Bacterial growth continued actively in the infected tracts of osteoblasts, while the enormously engorged macrophages described above gradually disappeared from these regions and probably disintegrated. By the 12th or 15th day the infected areas had often developed into large lumps, of a golden brown colour when seen by transmitted light, and which in fixed preparations were intensely acid-fast. Some of these lumps were serially sectioned and were found to consist of an almost solid mass of bacilli, the growth of which had probably disrupted the cells. In spite of this relatively enormous infection, however, the osteoblasts surrounding the lump of bacteria continued to grow vigorously even when the organisms belonged to a virulent strain, and heavily infected osteoblasts have been seen in all stages of mitosis after 21 days' cultivation *in vitro*. In the control lung cultures the infection remained under control throughout the maximum culture period (21 days).

It was interesting that actively proliferating tissue had little or no inhibitory effect on the growth of free bacteria in its immediate neighbourhood. In Exp. 5 the bacteria deposited on the osteoblast cultures, which had been grown for six passages before infection, multiplied even more luxuriantly than in the control droplets of suspension placed on a plasma and embryo extract clot without tissue, suggesting that the presence of growing tissue may even promote bacillary multiplication provided phagocytosis is restricted.

Conclusions (§ II)

(1) The wandering cells by their superior phagocytic activity protect the tissue cells from heavy intracellular infection.

(2) This protective action is limited by three factors: (i) the density of infection relative to the volume of the explant, (ii) the growth-rate of the infecting strain, (iii) the proportion of wandering cells which the tissue contains.

(3) When protection by wandering cells is inadequate, the tissue cells (osteoblasts and fibroblasts) ingest many bacilli. Bacterial growth in the tissue is then uncontrolled and finally disrupts the culture, whether the infecting strain is virulent or avirulent.

(4) Mitotic division remains active in the presence of heavy uncontrolled infection by a virulent strain of bacilli.

(5) Rapidly growing tissue does not inhibit and may even promote the multiplication of neighbouring free bacilli.

III. *The viability of bacilli phagocyted by wandering cells*

As described in § II, embryonic lung cultures could control an infection of avian tubercle bacilli for a limited period (2–3 weeks), after which the bacterial content of the tissue usually increased rapidly. The observations showed that bacilli not only survived but multiplied in the fibroblasts, but to what extent organisms phagocyted by wandering cells survived was less certain. Experiments were therefore made on lung cultures in which bacterial growth was still under control, to test the viability of the bacilli imprisoned in wandering cells.

The first experiments were simple and consisted in leaving lung cultures, which had been subcultivated normally for various periods, without change of culture medium. After some days the wandering cells began to degenerate, though some survived for a week or more. As cell degeneration progressed, the ingested bacilli began gradually to multiply in the cytoplasm which became more and more distended and finally disintegrated. The same result could be achieved by exposing the cells for a few minutes to strong light which killed the macrophages but not the phagocyted organisms; the whole process was observed on the warm stage of the microscope, from the degeneration of the infected wandering cells in response to intense light up to the disruption of the cytoplasm by the slowly proliferating bacteria it contained.

A much more rapid and precise method for testing the viability of the ingested bacilli was then developed.* It has been described in detail in the section on technique, and consists of drying the infected tissue cultures and then incubating them face downwards on a Löwenstein plate. This treatment preserves the gross structure of the cells, and in stained preparations fibroblasts and wandering cells are easily distinguished apart even after several days' incubation on the plates. The method shows clearly whether an early bacterial colony is

* A modification of this method has also been successfully applied to sputum smear cultures by Brieger and Boot; the results are being described in a separate publication.

derived from intracellular or free organisms. Experiments were made with all five strains of bacilli on tissue cultures grown for 2–21 days; the dried cultures were kept on Löwenstein's medium for 1–14 days.

The results showed that at least a large proportion of the phagocyted bacilli survived even in lung cultures grown for 21 days; in every culture examined after the treatment described above, many beautiful little colonies of bacilli were seen sprouting actively from the dead wandering cells and fibroblasts after 3 days' incubation on Löwenstein's medium (Pl. 3, figs. 10–15). It is noteworthy that the ingested bacilli grew far more rapidly when the dead tissue culture was incubated on a Löwenstein plate than they did in the untouched tissue culture medium after the infected cells had been allowed to degenerate spontaneously or had been killed by strong light.

These observations referred to cultures which had received a fairly massive infection, and an experiment was made to find whether phagocyted bacilli would survive in more lightly infected tissue. For this purpose we used some of the cultures treated with bacillary suspensions of the W strain, diluted respectively to 10 and 1 % of the usual concentration (see p. 364). On the 3rd, 6th and 12th day, cultures from both series were cut in half, and 3 days later, i.e. after 6, 9 and 15 days' growth (one, two and four passages), one of each pair was dried and placed on a Löwenstein plate, while the other was fixed and stained as a control. Even after four passages (15 days) profuse bacterial growth from the infected wandering cells and fibroblasts was obtained in both sets of cultures.

The character of the new bacillary growth varied somewhat according to the bacillary strain with which the lung culture had been infected. The early stages in the development of the new colonies were studied in cultures infected with each of the five strains. For these observations the infected lung explants were grown for 9–12 days before being dried and incubated on Löwenstein's medium for different periods. They were then fixed, stained and examined.

The most spectacular form of growth was provided by the phagocyted bacilli of the avirulent AF III strain. In control cultures fixed alive on the 9th day of cultivation in plasma and embryo extract, the ingested organisms appeared as rods and filaments of widely different lengths and degrees of acid-fastness, some of which had small side branches (Pl. 3, fig. 14). After the culture had been dried and incubated for 1 day on Löwenstein's medium, filamentous bacilli, some acid-fast but most nearly colourless, protruded in all directions from the dead host cells. During the 2nd day these filaments rapidly lengthened, multiplied and became more acid-fast, though non-acid-fast organisms remained throughout the culture period and often formed complete colonies. At the same time the filaments began to develop many side branches, and by the end of the 3rd day the infected macrophages presented an astonishing sight (Pl. 3, fig. 15). The nucleus and cytoplasm of the dead cells were often clearly visible, but permeating the cell body and spreading outwards through the surface membrane was a miniature mycelial network of exquisite delicacy. After 6 days' incubation some of the peripheral colonies had begun to break down into rods, but others showed the mycelial type of growth in an even more exaggerated form. The breakdown of the branched filaments continued, and in one culture which had been kept on Löwenstein's medium for 14 days and then transferred to a fresh plate for 4 days, all the colonies consisted of rods and short filaments. Subsequent investigation has shown that this is the normal life history of this strain.

Ingested bacilli of the avirulent L strain grew in a rather similar way to those of the AF III strain. In the 9-day control cultures the phagocyted rods varied greatly in length and acid-fastness. When the cultures were dried and placed on Löwenstein's medium the bacilli in the dead wandering cells elongated, proliferated and showed the same variation in staining reaction. Branched forms were common by the 3rd or 4th day but were fewer than in the AF III cultures in which nearly all the filaments were branched. Some of the filamentous colonies broke down into rods, but by the 8th day (the oldest stage examined) the culture was still very heterogeneous with colonies consisting of a mixture of short rods and filaments, both branched and unbranched, of various lengths.

The bacilli of the three virulent strains, i.e. the B, W and O, differed little from each other when the infected lung cultures were killed and incubated on Löwenstein plates. In 12-day control cultures infected with the highly virulent B strain (Pl. 3, fig. 12) the ingested bacilli appeared as small acid-fast rods densely packed in the macrophages. After drying and 1 day's incubation on Löwenstein's medium, the dead, infected cells had become much distended by the multiplication and elongation of the rods, whose appearance was much more homogeneous than in similar experiments with the AF III and L strains. The tiny colonies rapidly enlarged (Pl. 3, fig. 13), and soon burst and engulfed the corpses of the host cells. In some colonies the filamentous form persisted for 15 days, but in others the filaments had broken down into small rods by the 4th or 5th day (cf. the 'standard life cycle' in embryo extract, Brieger & Fell, 1945). A branched organism was seen once only, in a culture grown on Löwenstein's medium for 6 days.

Cultures infected with the virulent W and slightly virulent O strains behaved in a similar way but grew almost entirely as short, unbranched filaments without reverting to the small rod form during the period of incubation (cf. 'raft colonies', Brieger & Fell, 1945). Though most of the bacilli were acid-fast, non-acid-fast elements were fairly numerous.

Conclusions (§ III)

(1) Both virulent and avirulent bacilli ingested by wandering cells may survive in the cells for at least 21 days.

(2) If the host wandering cell is killed, the phagocyted bacilli are capable of immediate and profuse growth on suitable nutritive medium.

(3) The results provide no evidence that ingested bacilli of either virulent or avirulent strains are damaged by the phagocytosis.

DISCUSSION

The most remarkable feature of the results described above is the symbiosis which was established between the embryonic chicken cells and avian tubercle bacilli of not only avirulent but also highly pathogenic strains. In our experiments with embryonic lung explants, a balanced relationship between the tissue and the infection was maintained for at least 2–3 weeks; during this period the tissue inhibited or greatly retarded the multiplication of the bacilli, but did not destroy them, while, on the other hand, the most virulent organisms inflicted no obvious damage on the cells.

This temporary control of the infection by the explants was due to the wandering cells which rapidly engulfed the free bacilli and suppressed their growth. Fibroblasts, epithelial cells or osteoblasts, which also ingested bacilli, could not restrict the intracellular multiplication of the organisms to the same extent, but to a varying degree these cells were protected from infection by the vastly superior phagocytic activity of the wandering cells. This protection was limited by the following factors: the density of the infection relative to the size of the explant, the growth-rate of the bacilli and the proportion of wandering cells which the tissue contained. Thus when a very small lung explant or a culture of osteoblasts containing relatively few wandering cells was heavily infected with a rapidly growing strain of bacilli, the macrophages were too few to cope with the infection; consequently, the fibroblasts and osteoblasts ingested many organisms which multiplied in their cytoplasm so that the infection in the tissue was not controlled but steadily increased.

As stated above, in most cultures, even under the most favourable conditions, control of infection was only temporary, and we never observed the complete disappearance of the bacilli described by Maximow (1924) and by Timofejewsky & Benewolenskaja (1925) in cultures from a resistant host infected with a virulent but non-pathogenic strain. This loss of control in our cultures was largely due to the gradual disappearance of the wandering cells during repeated subcultivation. After 2–3 weeks the fibroblasts became ever more densely infected, free bacilli reappeared and the balanced relationship between the cells and the bacilli was disturbed in favour of the latter. Even when infected with bacilli of a very pathogenic strain, however, our material never showed the rapid, widespread necrosis observed by Maximow in cultures of adult rabbit tissue infected with bovine bacilli nor even the less drastic degeneration of parenchymatous cells described by Timofejewsky & Benewolenskaja in human embryonic tissue infected with a virulent human strain. In our 42-day embryonic lung cultures, infected with a strain of proved virulence, fibroblasts packed with bacilli not only appeared undamaged but might undergo apparently normal mitotic division, and the histological picture resembled that described by Maximow (1928) in rabbit tissue cultures infected with the BCG strain. The bacilli seemed eventually to kill the host cell merely by proliferating in the cytoplasm until they burst the cell membrane. The fate of bacilli ingested by multinucleate giant cells is not so readily studied in cultures of chick embryonic tissue as it is in those of adult material, and will therefore be considered in a later publication.

The question arises as to whether the symbiosis we have described is peculiar to embryonic chicken tissue or whether it appears in cultures of adult tissue also. Preliminary experiments with infected cultures of adult cock's spleen have not indicated any greater susceptibility of the adult cells to a moderately virulent avian infection. Viability tests of the type described on p. 360 have shown that the revival of ingested bacilli is as dramatic in the adult wandering cells as it is in those of the embryo, large colonies developing from the dead macrophages in adult spleen cultures killed 18 days after infection (the longest period so far investigated).

Thus our observations on virulent infection of the avian type in avian tissue cultures differ from those of previous authors on virulent infection of the mammalian type in cultures of mammalian tissue from either a susceptible or a resistant host. A possible explanation of this discrepancy might be found in the specific nature of avian infection *in vivo*. It is known that while the spontaneous infection of chickens by avian bacilli may produce the familiar picture of a generalized tuberculosis with caseous foci (Feldman, 1938), experimental infection of both chickens and young rabbits usually evokes the Yersin type of reaction

with septicaemia but no macroscopic evidence of caseation. The histological picture of the spleen in avian infection of the Yersin type is very reminiscent of that seen in our tissue cultures.

Until the observations on mammalian tissue cultures infected with pathogenic mammalian strains have been reinvestigated, however, it is impossible to offer more than the most tentative explanation of the differences between our results with the avian type and those of Maximow and of Timofejewsky & Benewolenskaja with the bovine and human types.

It remains to consider whether the balanced relationship between phagocyted bacilli and host cells which has been demonstrated in our cultures also occurs *in vivo*. A similar symbiosis between macrophages and tubercle bacilli has been shown in animals infected with a strain to which they are naturally resistant (Dembinski, 1899; Stanley Griffith, 1930), but whether such a condition exists in pathogenic infection is not yet clear. It is generally believed that in progressive infection the bacilli multiply in the cells and destroy them, while in regressive phases of infection the bacilli are destroyed by the host cells (Robertson, 1941). The experimental evidence is still contradictory. While Metschnikoff (1905) stated that he never observed the destruction of ingested tubercle bacilli in susceptible species, Kahn, Hotopp & Schwartzkopf (1936) report that human tubercle bacilli are destroyed when ingested by guinea-pig histiocytes and cannot be revived by culture on egg medium. According to Sabin & Doan (1927) bacilli survive in histiocytes but are destroyed by clasmatocytes. The observations of Lurie (1944) throw a new light on the possibility of a balanced host:parasite relationship at least in the initial stages of infection; he found that bovine tubercle bacilli ingested by macrophages of the rabbit spleen were alive 2 days after injection, and both cells and bacilli multiplied when the infected tissue was transplanted into the anterior chamber of a rabbit's eye. The question of the intracellular digestion of tubercle bacilli in immunized tissue is even more obscure but is beyond the scope of the present communication.

The tubercle bacillus, like that of leprosy, may be one of those organisms that are resistant to intracellular digestion. If the symbiosis between macrophages and bacilli that we have seen in our cultures proves to be a common feature of certain phases of tuberculous infection it would help to explain the period of initial latency in human infection or the fact that a one-germ infection can kill a guinea-pig (Wamoscher, 1927). Similarly, the sudden onset of the disease after trauma might be due to the liberation of ingested bacilli into the favourable medium provided by the haematoma.

Many more experiments on explanted tissue infected both *in vivo* and *in vitro* must be made, however, before conclusions can be safely drawn about the general applicability of the observations we have described.

SUMMARY

1. Explants of embryonic chick tissue were infected with avian tubercle bacilli of different degrees of virulence to study (*a*) some of the factors on which control of infection in tissue cultures depends and (*b*) the viability of bacilli ingested by wandering cells.

2. Embryonic lung cultures could control infection for 2–3 weeks owing to the intense phagocytic activity of the many wandering cells which they contained. The tissue appeared unharmed by infection with bacilli of either virulent or avirulent strains.

3. The wandering cells largely protected the much less phagocytic fibroblasts from intracellular infection.

4. This protective action of wandering cells towards less phagocytic cell types was limited by three factors: (1) the density of the infection relative to the volume of the explant, (2) the growth-rate of the infecting strain and (3) the proportion of wandering cells which the tissue contained.

5. Even under the most favourable conditions control of bacillary growth in the tissue was only temporary, largely owing to the gradual disappearance of the wandering cells during repeated subcultivation.

6. As control of bacillary growth was lost, the lung fibroblasts became packed with bacteria, but they appeared undamaged and might even undergo normal mitotic division. Eventually the ingested bacilli seemed to kill the cells by proliferating until they burst the cytoplasm.

7. Embryonic avian tissue cultures though infected with a highly virulent strain never showed the rapid, widespread necrosis observed by Maximow in cultures of adult rabbit tissue infected with a virulent bovine strain. Possible explanations of this disparity are discussed.

8. A special method was devised for testing the viability of bacilli phagocyted by macrophages. It showed that phagocyted bacilli, whether virulent or avirulent, survived for at least 21 days; when the host wandering cells were killed the ingested organisms were capable of immediate and profuse growth on suitable nutritive medium.

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EXPLANATION OF PLATES

Note. All the preparations figured were fixed in Zenker's fluid, stained with carbol fuchsin and Ehrlich's haematoxylin and mounted whole. *b*, bacilli; *bo*, bone; *c*, cytoplasm; *ep*, epithelium; *ex*, explant; *f*, fibroblast; *i.f.*, infected fibroblast; *i.m.*, infected macrophage; *m*, macrophage; *n*, nucleus.

The photomicrographs were made by Mr V. C. Norfield, head assistant at the Strangeways Research Laboratory.

PLATE 1

Fig. 1. Large explant of embryonic lung fixed 24 hr. after infection with a suspension of the highly virulent B strain. Macrophages, many of them packed with bacilli, are wandering into the droplet of suspension. Some spindle-shaped fibroblasts have also migrated from the explant. (× 215.)

Fig. 2. Similar infected explant from the same experiment fixed after 3 days' growth. The tissue has now spread over the infected area, and nearly all the free bacilli have been phagocyted. Note the compact groups of engorged macrophages embedded in the fibroblastic tissue. The fibroblasts contain few bacilli. (× 215.)

Fig. 3. A small, infected explant from the same experiment fixed after 3 days' growth (cf. the large, diffuse mass of infection with the small, compact groups of engorged macrophages shown in fig. 2). Both macrophages and fibroblasts are heavily infected; since the small explants contain much fewer wandering cells than the large cultures, the bacilli have time to multiply extensively before being engulfed. (× 215.)

Fig. 4. A small culture from the same experiment fixed after 6 days' growth. A fibroblast, though heavily infected with the virulent B strain, has undergone a normal mitotic division and is shown in late telophase. (× 1200.)

PLATE 2

Fig. 5. Culture of embryonic lung fixed 42 days after explantation and infection with the virulent W strain. At this stage of cultivation few macrophages remain in the tissue, there is no longer any control of the infection and the fibroblasts are packed with bacilli. In spite of the dense and virulent infection, the culture continues to grow quite actively. (× 27.)

Fig. 6. High-power view of the field marked with a square in fig. 5. The amoeboid fibroblasts are crowded with virulent bacilli. (× 550.)

Fig. 7. Culture from the same experiment fixed after 42 days' growth. Although packed with virulent bacilli, the fibroblasts can still undergo normal mitotic division, as illustrated by the cell in metaphase. (× 1600.)

Fig. 8. Culture of embryonic frontal bone fixed 9 days after explantation and infection with the slightly virulent, actively growing O strain. Note the heavy, diffuse, uncontrolled infection in the tissue where the osteoblasts are packed with bacilli. (× 53.)

Fig. 9. Culture of embryonic lung from the same experiment, fixed after 9 days' growth. The infection is confined almost entirely to the macrophages, which are seen scattered in the fibroblastic tissue, and has not greatly increased as it has in the bone culture (cf. fig. 8). (× 53.)

PLATE 3

Fig. 10. Culture of embryonic lung fixed 12 days after explantation and infection with the highly virulent B strain. With rare exceptions all the bacilli are intracellular and no bacillary colonies are seen. (× 27.)

Fig. 11. Infected culture from the same experiment dried after 12 days' growth and then incubated for 3 days on a Löwenstein plate. The whole culture is peppered with small bacillary colonies derived from the phagocyted bacteria (see fig. 13). (× 27.)

Fig. 12. Infected culture from the same experiment dried after 12 days' growth and then fixed as a control. A macrophage killed by drying is seen. Note the many rod-shaped bacilli in the cytoplasm and the pcynotic nucleus. (× 1600.)

Fig. 13. The same culture as that shown in fig. 11. Note the bacilli actively sprouting from a dead macrophage (cf. fig. 12). (× 1600.)

Fig. 14. Culture of embryonic lung fixed 6 days after explantation and infection with the avirulent AF III strain. A macrophage containing a slightly branched filamentous bacillus is seen. (× 1600.)

Fig. 15. Infected culture from the same experiment dried after 6 days' growth and then incubated for 3 days on a Löwenstein plate. Branching mycelial filaments protrude in all directions from a dead macrophage. (× 1600.)

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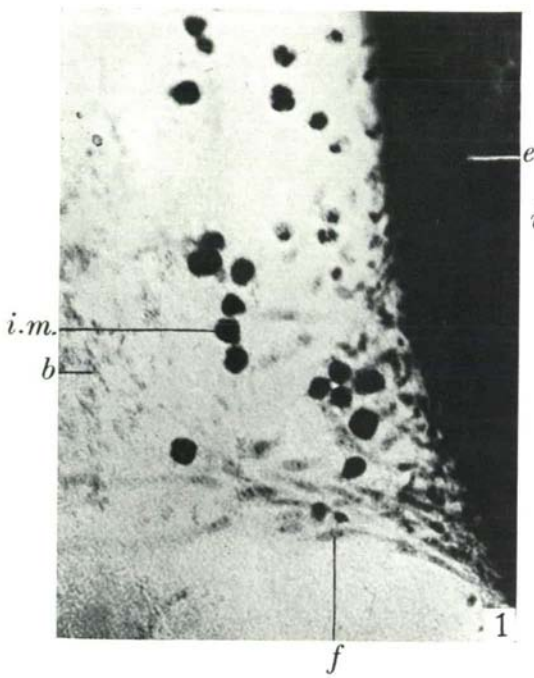

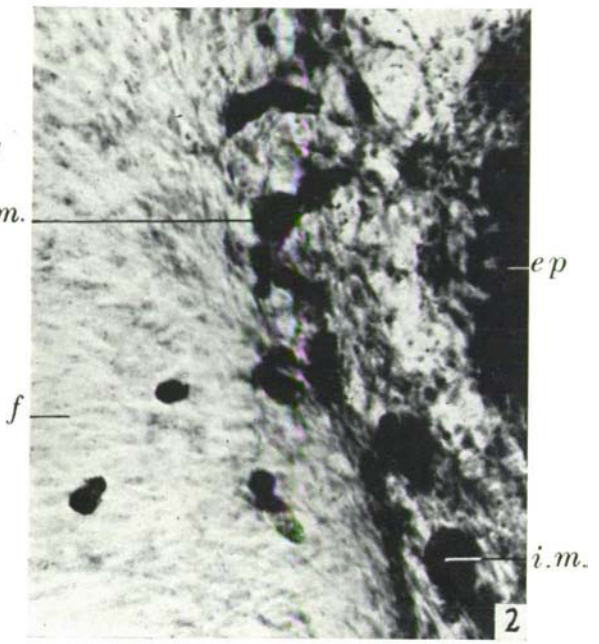

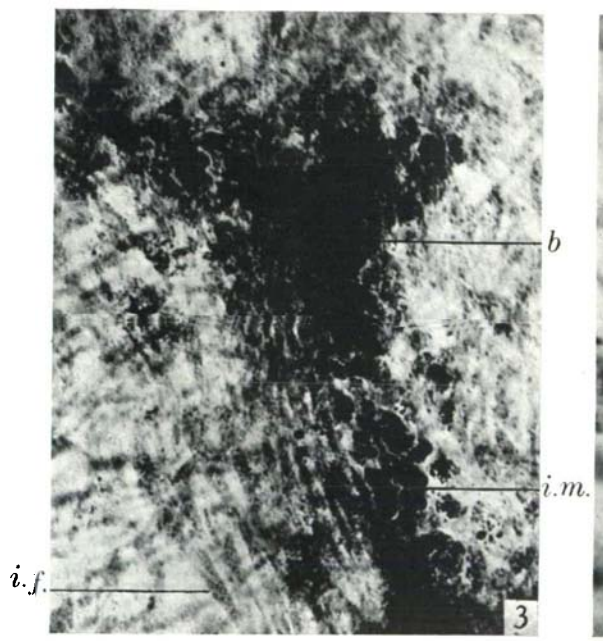

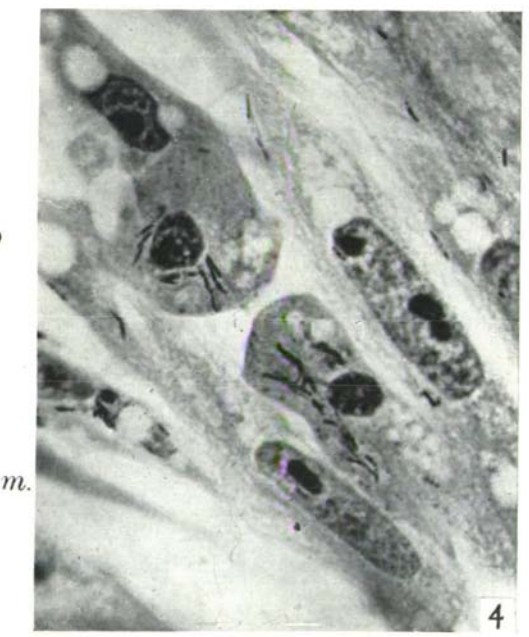

Figs. 1–4

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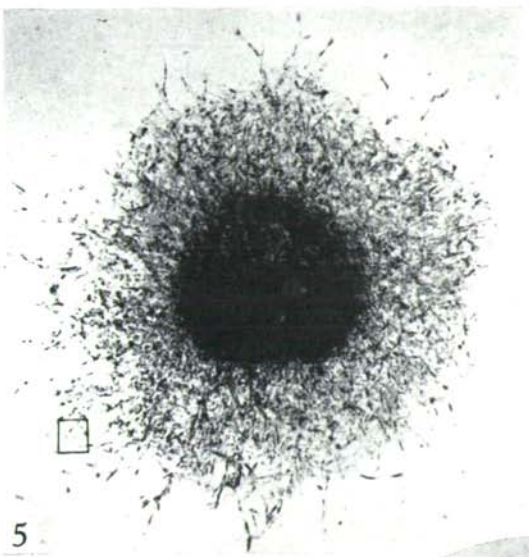

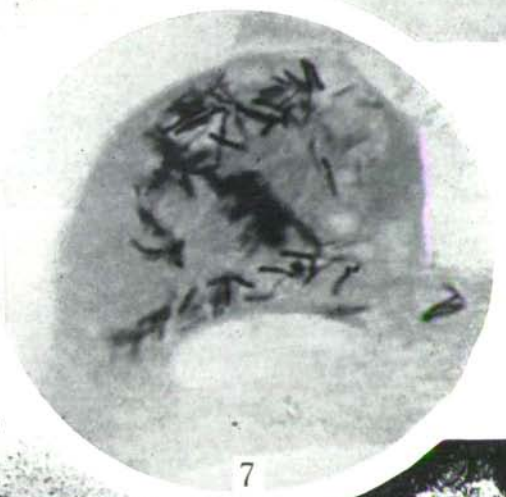

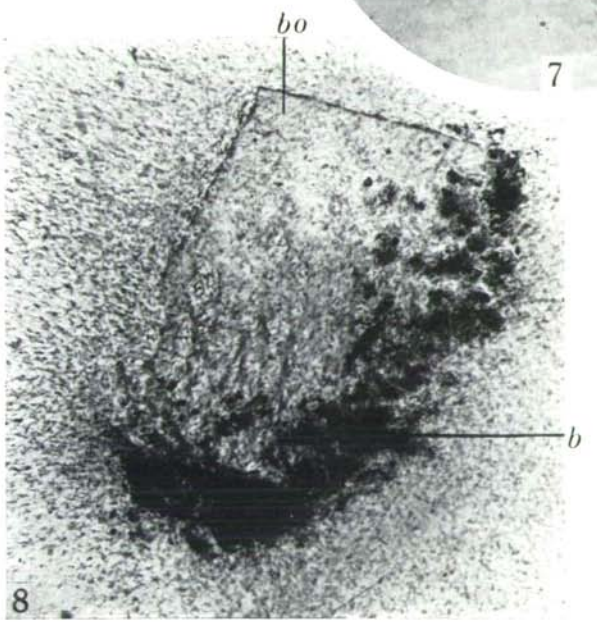

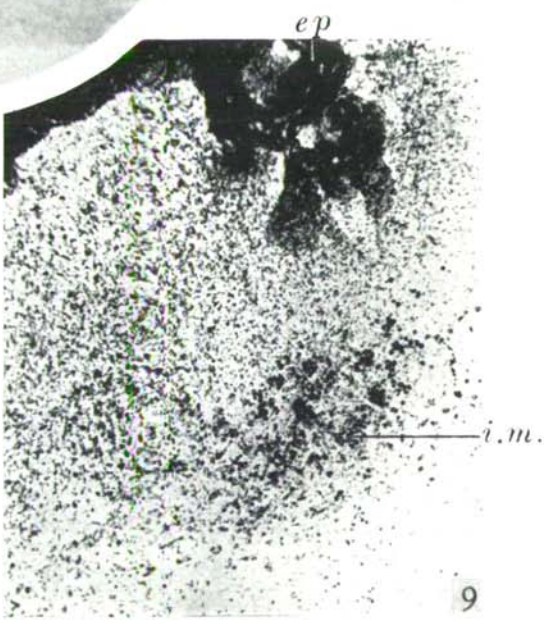

Figs. 5–9

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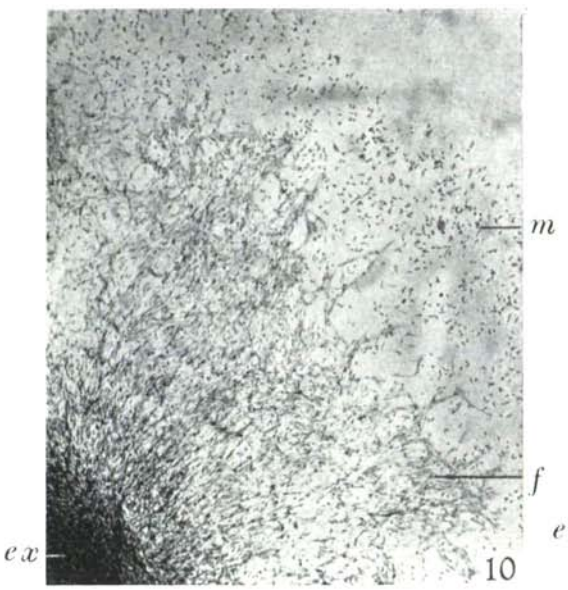

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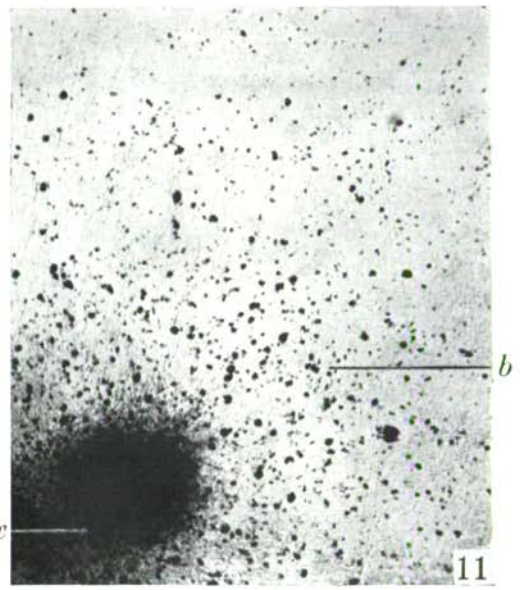

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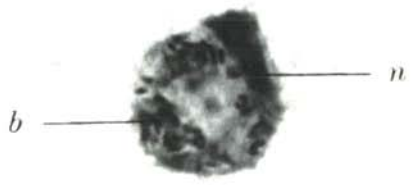

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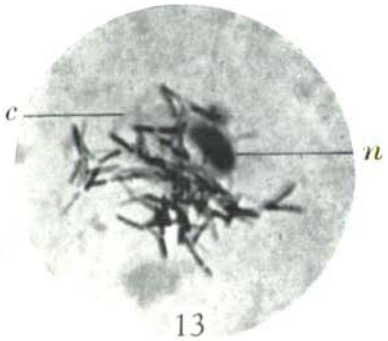

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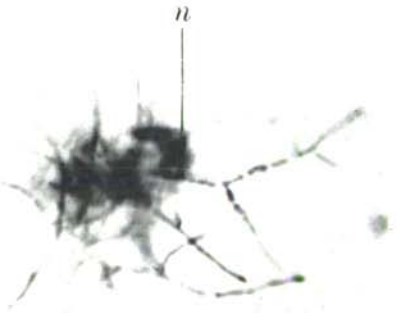

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Figs. 10–15

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